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14. ABSTRACT

The goal is to study autonomic biomarkers for an animal model of gulf war syndrome and to evaluate the effects of treatments. During the first year, progress has been made on acquisition and training of personnel, setup of methodologies and completion of the initial experiments. Experiments tested the effect of low dose sarin in mice on 1) central aminergic brain systems; 2) peripheral autonomic nervous system using molecular markers; 3) autonomic cardiovascular and cardiac function using radiotelemetry and echocardiography and 4) effect of treatment with memantine on blood pressure and cardiac response to sarin. Results show that low dose sarin elicits prominent region specific effects on brain dopamine systems which may bear relevance to the behavioral changes associate with nerve gas exposure. Low dose sarin produced significant effects on cardiac autonomic balance without producing changes in heart function. There were interactions between memantine and sarin as seen by blood pressure and cardiac function. Work is underway to explore the effects of other drug treatment which are known to be effective in modulating autonomic function.

15. SUBJECT TERMS

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1. Introduction

The project goal is to study autonomic biomarkers for an animal model of gulf war syndrome and to evaluate the effects of treatments. During the first year of the project, progress has been made on the acquisition and training of personnel, the setup of methodologies and the completion of the initial phase of the experiments. The experiments tested the effect of low dose sarin in mice on 1) central aminergic brain systems; 2) peripheral autonomic nervous system using molecular markers; 3) autonomic cardiovascular and cardiac function using radiotelemetry and echocardiography and 4) effect of treatment with memantine on blood pressure and cardiac response to sarin. Results show that low dose sarin elicits prominent region specific effects on brain dopamine systems which may bear relevance to the behavioral changes associate with nerve gas exposure. Low dose sarin produced significant effects on cardiac autonomic balance without producing changes in heart function. There were interactions between memantine and sarin as seen by blood pressure and cardiac function. Work is underway to explore the effects of other drug treatment which are known to be effective in modulating autonomic function.

2. Body

Experiment 1: Determination of the effect of low dose sarin on the central nervous system in mice.

Key Finding: Low dose sarin produces specific changes in brain dopamine which may relate to the behavioral and neurotoxic effects.

In order to understand the effect of low dose exposure to sarin on physiological and behavioral functions, we analyzed the levels of catecholamines and their metabolites [dopamine (DA), dihydroxyphenyl-acetic acid (DOPAC) and homovanillic acid (HVA)] in different brain areas after exposure of mice to sublethal doses of sarin. Data shows that a low dose of sarin has potent, long-term, region specific effects on brain catecholamine systems.

Experimental Details: Male C57BL/6 mice were obtained from Harlan Laboratories weighing 20-25g, aged 3 months. Mice were housed in individual cages on a 12:12h light/dark cycle. A standard pellet diet and water were available ad libitum. All mice were allowed 7 days of acclimation to facilities and subjected to at least 3 days of handling before dosing. All procedures were approved by the Laboratory Animal care and Use Committee of Wright State University, Dayton, OH.

Sarin (USAMRICD, Aberdeen Proving Ground, MD) was diluted in 0.9% saline to concentrations such that injection of 0.5 ml/100g would provide a dose of 8 μg/kg (0.05 X LD_{50}) or 64 μg/kg (0.4 X LD_{50}). Two groups of mice were injected subcutaneously once a day for 2 days with saline or sarin (0.05 LD_{50} or 0.4 LD_{50}). Mice were sacrificed at 4 and 8 weeks post injection. After removal of frontal cortex (FC), the brain was sliced into 2mm slices with removal of amygdala and caudate nucleus using standard markers.

Tissues were stored at -80°C until further processing . For catecholamine measurements, tissues were homogenized in 300µl of 0.2N $HClO_4$ and centrifuged to remove cellular debri.

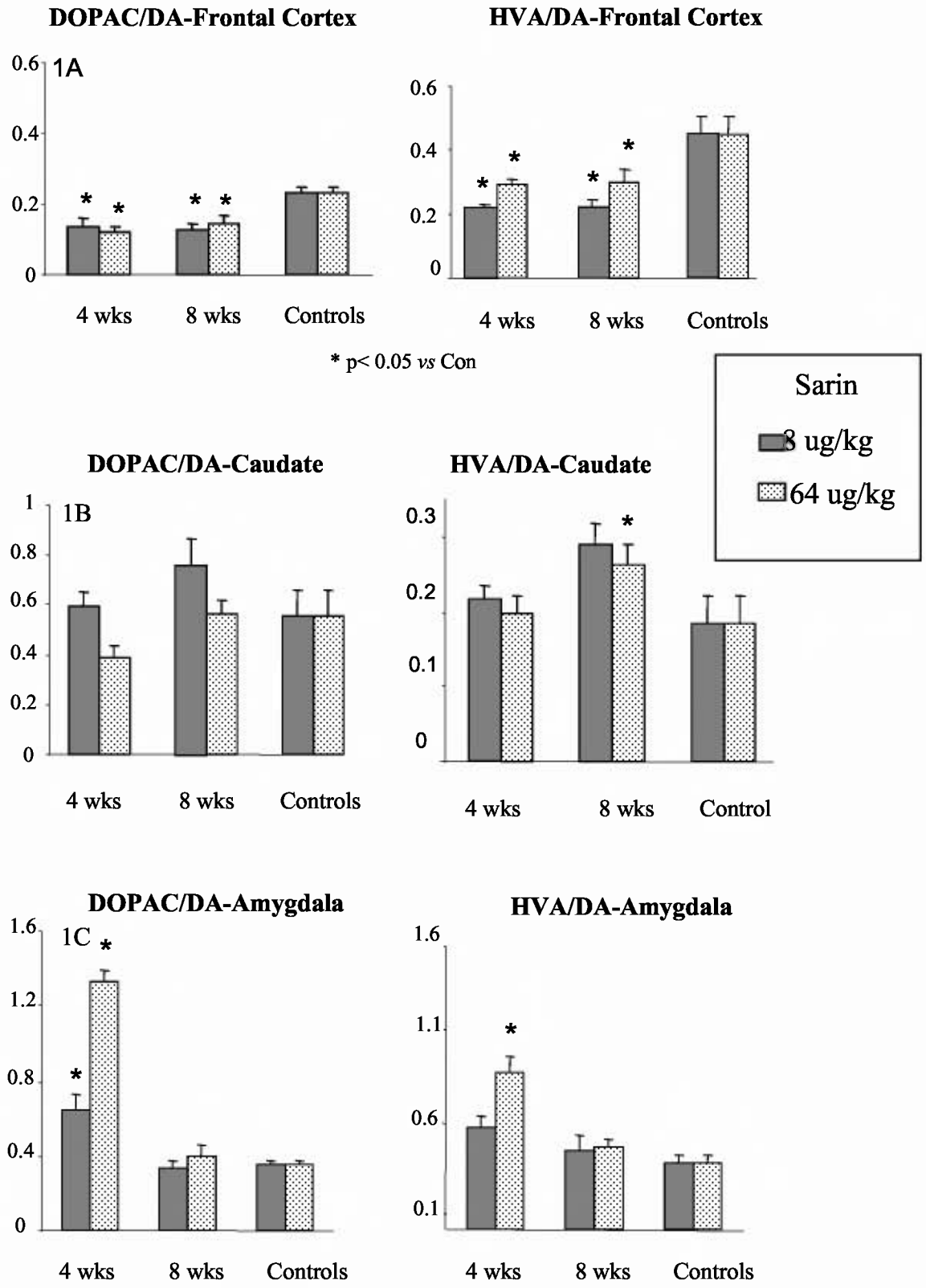
Catecholamine determinations: Norepinephrine (NE), dopamine (DA), dihydroxyphenyl-acetic acid (DOPAC), homovanillic acid (HVA) and serotonin (5-HT) were analyzed by High Performance Liquid Chromatography (HPLC BAS). Catecholamines were separated by C18 reverse phase column in acetate/citrate mobile phase. Amounts were determined by electrochemical amperometric detection at 0.8 volts.

Statistical Analysis: Data was analyzed by using STATISTICA (Statsoft, v6) data analysis software. One way ANOVA was used to evaluate overall significance.

Results: In both dose groups and time points, significant decreases in the DOPAC/DA ratio and HVA/DA ratio were observed in frontal cortex. There was an increase in the DOPAC/DA ratio and HVA/DA ratio in the amygdala for the 4 week group but not for the 8 week group at both doses. There was no significant change observed in the caudate. Levels of norepinephrine and serotonin were not changed in any of the brain regions (data not shown). Graphs presented below show the effects of sarin on catecholamine levels in cortex (1A), amygdala (1C), caudate (1B).

Discussion: The results indicate that low dose sarin produces long term changes (up to 2 months after injection) in cortical DA metabolism. The fact that DA increases whereas the metabolites, DOPAC and HVA, remain the same suggests a decrease in use of DA. Since the frontal cortex receives projections from A10 DA cell bodies, the data suggests changes in this pathway. In amygdala, DA levels remain the same; whereas, DOPAC and HVA levels increase. This suggests an increase in DA use, opposite to that seen in the cortex. These increased levels of DOPAC and HVA in the amygdala return to normal with time (8 week). The amygdala receives projections from A10 DA cell bodies as well as A8 DA cell bodies, which might account for the difference from the cortex. Sarin produced no significant change in the caudate nucleus, which receives projections from A9 DA cell bodies.

Conclusions: These data show that even low doses of sarin have potent, long-term effects on the catecholamine systems. Furthermore, these effects are brain region specific. This data will be presented at the 2009 Society of Toxicology Meeting and a manuscript is in preparation.


DOPAC/DA-Frontal Cortex
1A
0.6
0.4
0.2
0
4 wks
8 wks
Controls
HVA/DA-Frontal Cortex
0.6
0.4
0.2
0
4 wks
8 wks
Controls
* p< 0.05 vs Con
Sarin
8 ug/kg
64 ug/kg
DOPAC/DA-Caudate
1B
1
0.8
0.6
0.4
0.2
0
4 wks
8 wks
Controls
HVA/DA-Caudate
0.3
0.2
0.1
0
4 wks
8 wks
Control
DOPAC/DA-Amygdala
1C
1.6
1.2
0.8
0.4
0
4 wks
8 wks
Controls
HVA/DA-Amygdala
1.6
1.1
0.6
0.1
4 wks
8 wks
Controls

Experiment 2: Determination of the effect of low dose sarin on autonomic neurochemical expression in mice. The focus was on peripheral components of the autonomic nervous system, the adrenal gland and the superior cervical ganglia.

Key Finding: Exposure to low, non-symptomatic doses of sarin produced change in the peripheral autonomic nervous system.

Experimental Details: C57BL/6 male mice were injected with sarin on two consecutive days (8μg/kg, .05x LD_{50} sc or 64μg/kg, 0.4x LD_{50}; sc) Control mice were injected with isotonic saline. Mice were sacrificed at 4 or 8 weeks after treatment. Adrenal glands and superior cervical ganglia (SCG) were procured, fixed, and stored in a -80° freezer until processing. Tissues were sectioned at 30μm with a cryostat and placed on slides. Tissue sections on slides were processed using in situ hybridization (ISH) for quantification of mRNA levels for markers of the autonomic nervous system (ANS): tyrosine hydroxylase (TH), acetylcholine esterase (AChE), and choline acetyl transferase (ChAT). Sections were incubated with 35S labeled oligonucleotide probes specific for the mRNAs (~.5 x 106 cpm/100 μl). Following a series of washing steps, slides were processed using a Fuji phosphor imaging system. TH, AChE, and ChAT mRNA levels in control and sarin treated mice were compared.

Results:

Changes in Adrenal mRNA levels in sarin treated mice

Enzyme	Sarin Dose	4 weeks	8 weeks
AChE	.05 LD50	Decrease	Decrease*
	.4 LD50	Decrease	Increase*
TH	.05 LD50	Decrease*	Decrease
	.4 LD50	Decrease	Decrease
ChAT	.05 LD50	Decrease	Decrease
	.4 LD50	No change	Decrease

*statistically significant; AChE-acetylcholinesterase, TH-tyrosine hydroxylase, ChAT-choline acetyltransferase

Changes in SCG mRNA levels in sarin treated mice

Enzyme	Sarin Dose	4 weeks	8 weeks
AChE	.05 LD50	Decrease*	Decrease
	.4 LD50	Decrease	Decrease
TH	.05 LD50	Increase	Increase
	.4 LD50	Decrease	Increase
ChAT	.05 LD50	Decrease	Increase
	.4 LD50	Increase	Decrease

*statistically significant; AChE-acetylcholinesterase, TH-tyrosine hydroxylase, ChAT-choline acetyltransferase

Discussion: Results support the idea that low dose, non-symptomatic exposure to sarin has significant effects on the peripheral autonomic nervous system. These are seen as alterations in autonomic biomarkers in the adrenal gland and SCG. The effects are seen long term, up to 8 weeks after exposure. It is possible that the neurocellular and cardiovascular changes seen in the mouse model may be relevant to the pathophysiology of Gulf War Illness. This work was presented at the Biosciences Review in 2008.

Experiment 3

Determination of low dose sarin exposure and co-treatment with mematine on blood pressure and cardiac function using radiotelemetry and echocardiography. The idea behind using the echocardiography is that it will provide information on heart function without intervention. There was some delay in implementing the methodology because of availability of the equipment and approval by the animal care and use committee.

Mematine treatment was used with the goal of establishing whether this might alter autonomic function in the sarin treated mice. Mematine is approved for use to treat Alzheimer's disease. It is in a class of drugs called "uncompetitive low to moderate affinity N-methyl-D-aspartate (NMDA) receptor antagonist. Mematine works by suppressing the activity of the chemical messenger glutamate in the brain.

Experimental Details: C57BL/6 male mice were injected with sarin on two consecutive days (8μg/kg,.05x LD50 sc or 64μg/kg, 0.4x LD50; sc) Control mice were injected with isotonic saline. At 12 weeks following treatment, arterial telemetry probes were implanted into the aorta through the carotid artery. Telemetry measurements of blood pressure and heart rate were taken at week 13 following treatment. Echocardiography measurements were made using a Siemens 512 Seqouia echocardiography machine with a 15 MHz probe. After baseline measurements, osmotic pumps containing memantine pumps were implanted subcutaneously and memantine was administered for 4 days (5 mg/kg/day). Repeat echocardiographic and telemetry measurements were taken following memantine treatment. Spectral analysis was conducted on the baseline data and is in progress for the memantine phase. Mice were sacrificed and adrenal glands, superior cervical ganglia, and brainstems were collected.

Results

Radiotelemetry: There was no significant difference in mean arterial pressure or heart rate between sarin and control groups (Figure 2). Variance and its frequency domains were calculated using spectral autoregressive methods (Figure 3). Changes were noted for pulse interval (PI) but not for arterial pressure. Sarin treated mice showed a reduction in PI variance during the dark period, but not during the light. There were similar changes for the high frequency component which is consistent with previous results. Work is in progress to conduct the spectral studies on the memantine treated group to determine if the treatment restores autonomic balance.

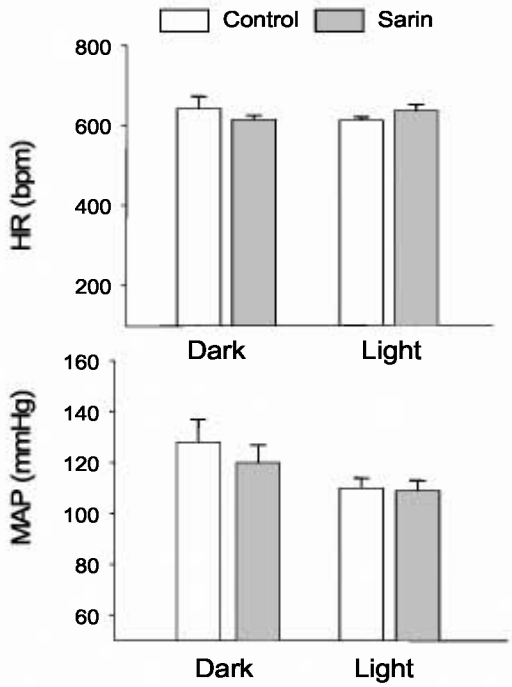


Figure 2: Dark and light measurements of BP and HR in sarin treated mice.

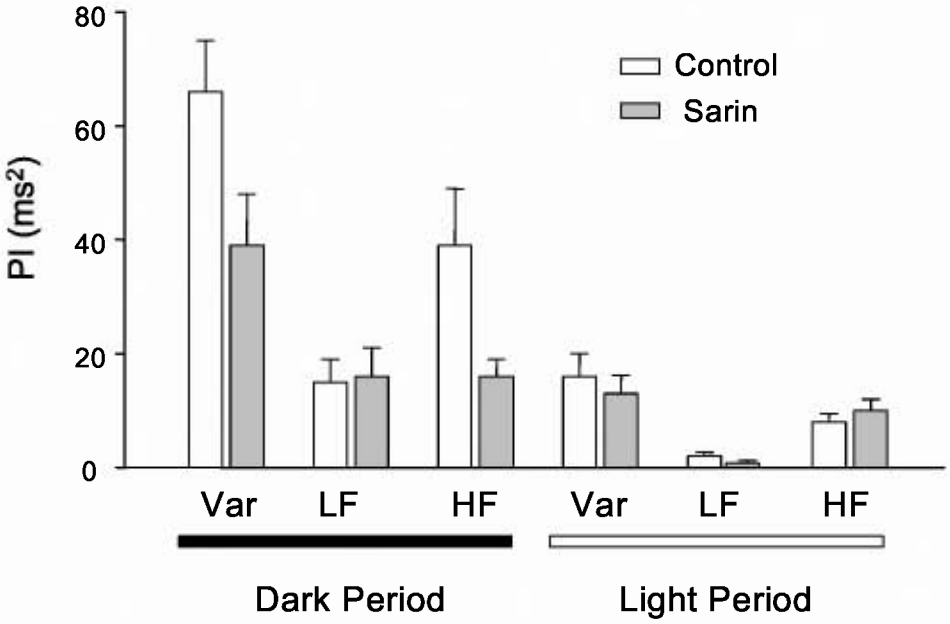
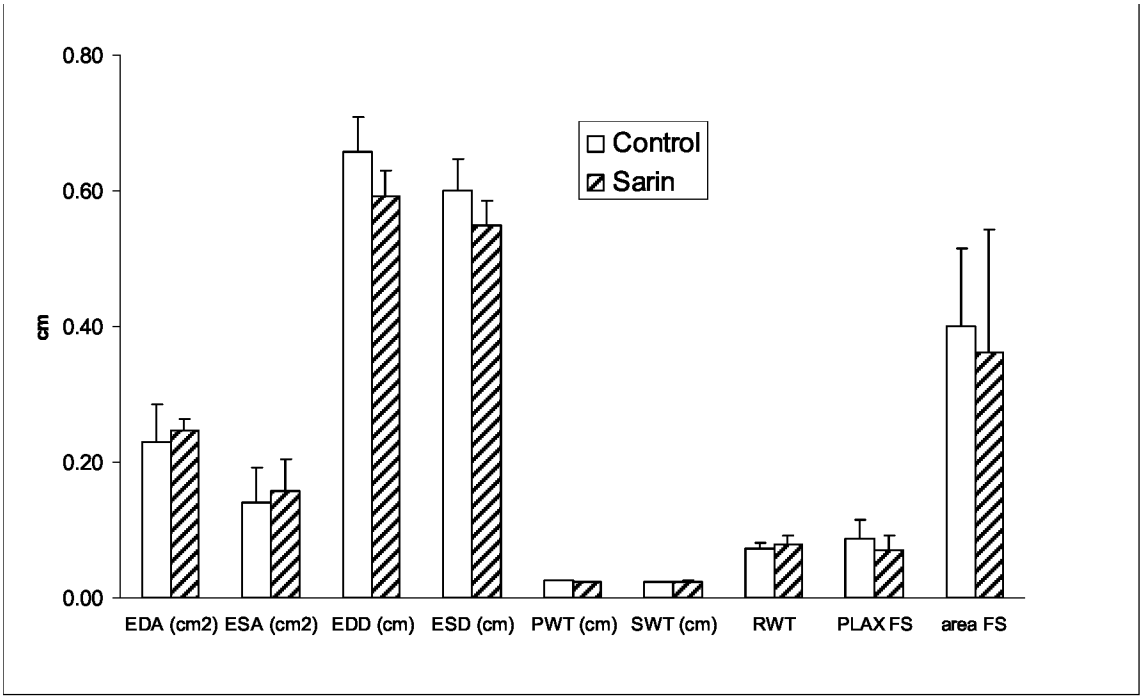


Figure 3: Spectral analysis of PI in sarin mice during the dark and light periods.



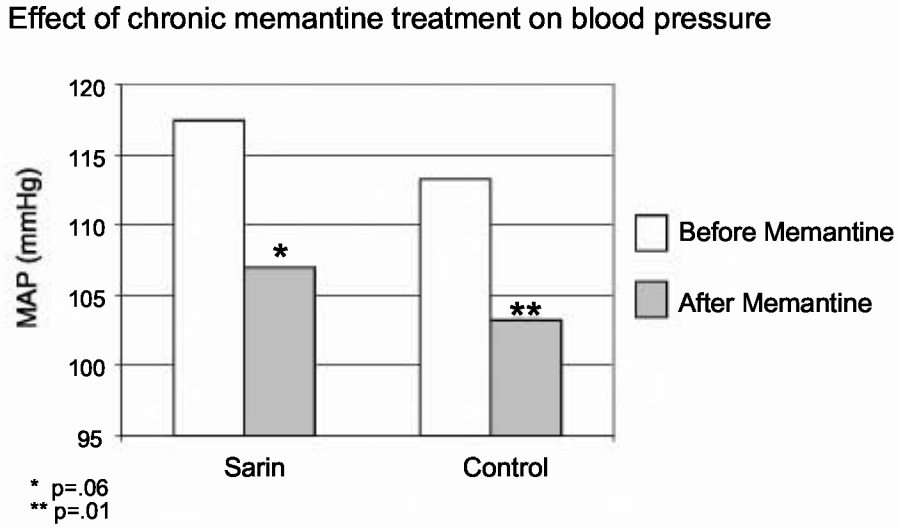
Abbreviation	Definition	Description
EDA	End Diastolic Area	Area of left ventricle at the end of diastole
ESA	End Systolic Area	Area of left ventricle at the end of systole
EDD	End Diastolic Dimension	Cross-sectional diameter of mid-left ventricle at the end of diastole
ESD	End Systolic Dimension	Cross-sectional diameter of mid-left ventricle at the end of systole
PWT	Posterior Wall Thickness	Thickness of left ventricle posterior wall
SWT	Septal Wall Thickness	Thickness of interventricular septal wall
RWT	Relative Wall Thickness	Ratio of total wall thickness to end diastolic dimension
PLAX	Posterior Long Axis	Echocardiographic sagittal view of the heart
FS	Fractional Shortening	Percentage shortening of specified dimension
EF	Ejection Fraction	Percentage of ventricular volume ejected during systole

Figure 4: Effect of sarin treatment on cardiac function as determined using echocardiography.

However, mean arterial pressure was significantly decreased in both Sarin-treated and control mice following Memantine treatment. Memantine did not have a significant effect on heart rate in either group.

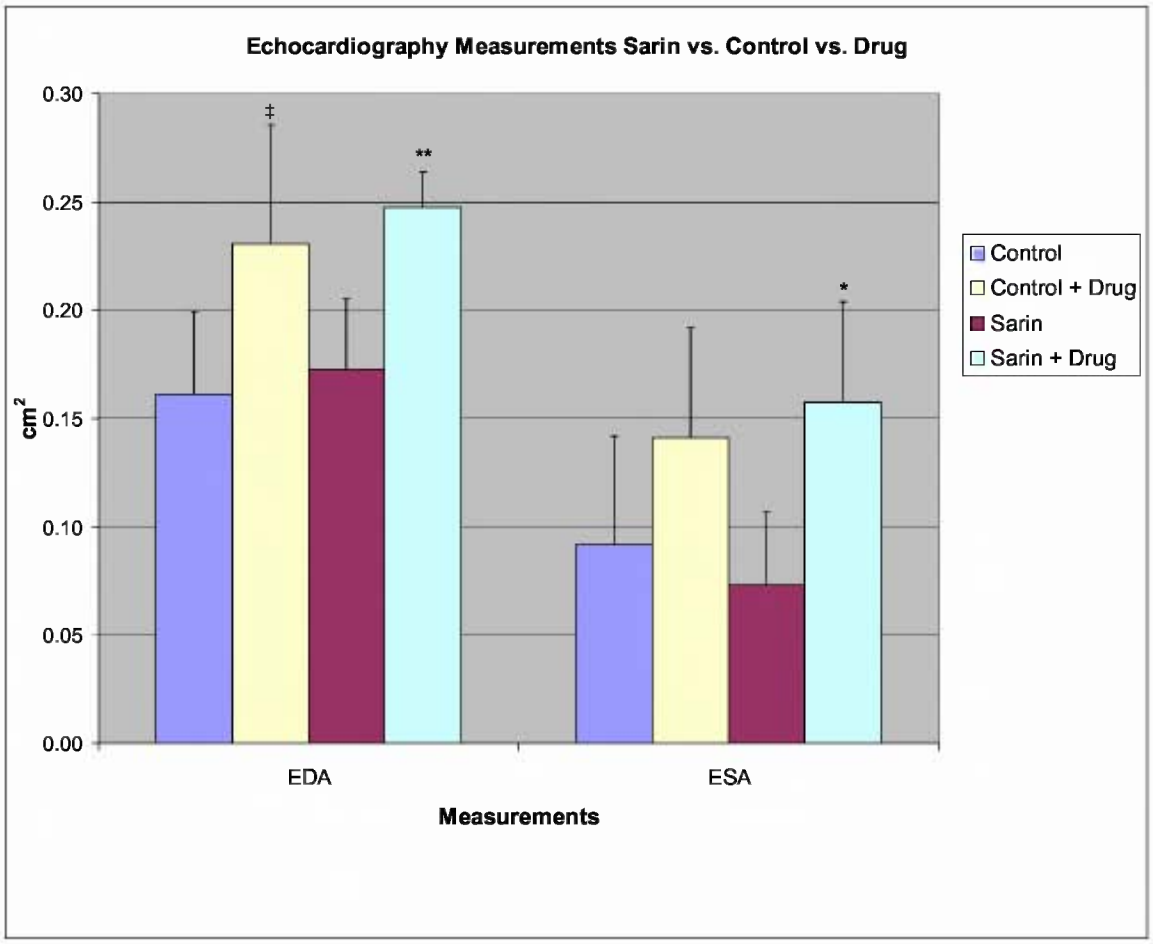
Effect of memantine on MAP

	Before Memantine	After Memantine	P value
Sarin	117.3 mmHg	106.9 mmHg	.06
Control	113.2 mmHg	103.2 mmHg	.01



Echocardiography: There was no significant difference between sarin and control groups. Mice pre-treated with sarin had significantly elevated EDA and ESA following treatment with memantine. In the sarin group, EF was significantly decreased following memantine treatment. Control mice also had elevated EDA and ESA, however, only the EDA elevation was statistically significant. ESD was significantly elevated in control mice. No significant changes were seen with other echo measurements.

	Before Memantine	**After Memantine**	**P value**
Sarin EDA ± SD (cm^2)	.17 ± .03	.25 ± .02	.003
Control EDA ± SD (cm^2)	.16 ± .04	.23 ± .06	.03
Sarin Ejection Fraction ± SD (%)	90 ± 8	69 ± 21	.05
Control ESD ± SD (cm^2)	.54 ± .05	.60 ± .05	.05



* p=.01 Sarin vs. Sarin + Drug
** p<.01 Sarin vs. Sarin + Drug
‡ p<.05 Control vs. Control + Drug

Discussion: Results show that a low dose of sarin has prominent autonomic effects even months after the exposure. However, this does not result in marked functional changes as measured with Although Sarin has been shown to alter heart rate variability and autonomic biomarkers, it does not appear to have a significant effect on clinical cardiac morphology or function. Echocardiography data suggests that memantine may alter cardiac function, increasing left ventricular volumes in end diastole and systole.

3. Key Research Accomplishments

1. Setup methods for chronic monitoring cardiovascular (radiotelemetry) and cardiac (echocardiography) function in mice. This provides the basis for the experimental goals.
2. Tested the effect of long term, low dose sarin exposure on brain dopaminergic systems. Results showed that there were tissue and level specific changes.
3. Tested the effect of long term, low dose sarin exposure on components of the peripheral autonomic nervous system. Results showed that there were limited changes.
4. Showed that autonomic cardiac function was reduced after low dose sarin exposure. Reduction in variance for heart rate is a pathological pattern which may have long term consequences.
5. Showed that low dose sarin exposure had little effect on heart function as evaluated using echocardiography. This suggests that the pumping capacity of the heart is not compromised.
6. Conducted a treatment test of a drug which is used in Alzhemier's disease, memantine. Results showed there were interactions with sarin in terms of cardiac function and blood pressure.
7. Presented findings at the Biosciences review meeting, sponsored by USAMRID.
8. Submitted an abstract for presentation at the Society of Toxicology meeting.

4. Reportable Outcomes

Dhawal, DP, Izu, B, Morris, M, Lucot JB, Low dose sarin exposure alters brain catecholamine neurochemistry, Presented Miami Valley Society of toxicology Meeting, 2008

Dhawal, DP, Izu, B, Morris, M, Lucot JB, Effect of low-dose sarin exposure on the neurochemistry of different brain structures in mice. Toxicological Sciences, in press, 2009

Izu, B, Lucot, JB, Morris, M,

5. Conclusions

The findings of the studies have importance in terms of terrorism and the effects of a chemical warfare agent on military and civilian populations. Our results show that a dose as low as 8 ug/kg in a mouse has significant effect on the brain and the autonomic nervous system. The effects are also long lasting which is important in terms of protection and treatment. For this series of experiments we tested the effect of memantine which is a blocker of glutamate receptors. It may be better to use the acetylcholinesterase inhibitors or perhaps other new compounds. The ultimate goal is to discover the mechanisms of the sarin induced pathologies and to find appropriate treatments.

'So what" – As mentioned above, the studies in mice have relevance to the human condition. Although it is difficult to test sarin exposure, accidental and terrorist incidences have occurred and there is evidence that some of the effects are neuropsychological in nature and very long lasting.